Whole blood resuscitation restores intestinal perfusion and influences gut microbiome diversity

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OBJECTIVE: Gut dysbiosis, an imbalance in the gut microbiome, occurs after trauma, which may be ameliorated with transfusion. We hypothesized that gut hypoperfusion following trauma causes dysbiosis and that whole blood (WB) resuscitation mitigates these effects.

METHODS: Anesthetized rats underwent sham (S; laparotomy only, n = 6); multiple injuries (T; laparotomy, liver and skeletal muscle crush injuries, and femur fracture, n = 5); multiple injuries and 40% hemorrhage (H; n = 7); and multiple injuries, hemorrhage, and WB resuscitation (R; n = 7), which was given as 20% estimated blood volume from donor rats 1 hour posttrauma. Baseline cecal mesenteric tissue oxygen (O_2) concentration was measured following laparotomy and at 1 hour and 2 hours posttrauma. Fecal samples were collected preinjury and at euthanasia (2 hours). 16S rRNA sequencing was performed on purified DNA, and diversity and phylogeny were analyzed with QIIME (Knight Lab, La Jolla, CA; Caporaso Lab, Flagstaff, AZ) using the Greengenes 16S rRNA database (operational taxonomic units; 97% similarity). α and β diversities were estimated using observed species metrics. Permutational analysis of variance was performed for overall significance.

RESULTS: In H rats, an average decline of 36% ± 3.6% was seen in the mesenteric O_2 concentration at 1 hour without improvement by 2 hours postinjury, which was reversed following resuscitation at 2 hours postinjury (4.1% ± 3.1% difference from baseline). There was no change in tissue O_2 concentration in the S or T rats. β Diversity differed among groups for all measured indices except Bray-Curtis, with the spatial median of the S and R rats more similar compared with S and H rats ($p < 0.05$). While there was no difference in α diversity found among the groups, indices were significantly correlated with mesenteric O_2 concentration. Members of the family Enterobacteriaceae were significantly enriched in only 2 hours.

CONCLUSION: Mesenteric perfusion after trauma and hemorrhage is restored with WB resuscitation, which influences β diversity of the gut microbiome. Whole blood resuscitation may also mitigate the effects of hemorrhage on intestinal dysbiosis, thereby influencing outcomes. (*J Trauma Acute Care Surg.* 2021;91: 1002–1009. Copyright © 2021 Wolters Kluwer Health, Inc. All rights reserved.)

KEY WORDS: Gut microbiome; trauma; hemorrhage; whole blood; rat.

The essential role of the gut microbiome on human health and disease has been well established. Composed of more than 1,000 different species, the gut microbiome is a dynamic network of commensal, symbiotic, and pathogenic microorganisms interacting to support the gut mucosal barrier, nutrient metabolism and absorption, and immune system homeostasis.[1,2] An imbalance in the composition and abundance of the gut microbiota, known as dysbiosis, is thought to account not only for various inflammatory and metabolic diseases but also for dysfunctions in the central nervous system via the gut-brain axis.[3]

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Research has shown that there is a loss of bacterial diversity and overgrowth of pathogenic bacteria in critically ill patients, believed to be associated with higher rates of hospital acquired infections, sepsis, and multiple organ failure.[1,4,5] Intestinal ischemia reperfusion has been implicated in the pathophysiology that contributes to these changes in the gut microbiome.[6] Hayakawa et al.[7] showed that dysbiosis occurs within hours of insult, with the depletion of traditionally beneficial bacteria (e.g., obligate anaerobes and *Lactobacillus*) and decreased levels of three major short chain fatty acids seen on the day of admission. Furthermore, in this same study, a shift toward a pathobiome was sustained during the first 14 days of admission, as the counts of pathogenic bacteria (*Enterococcus* and *Pseudomonas*) increased during the observation period and the levels of *Lactobacillus* and short chain fatty acids remained lower compared with controls.[7]

Similar outcomes in the changes of the gut microbiome composition have been observed following multiple injuries. An early clinical study showed phylogenic changes among the gut microbial population in critically injury patients, with a significant enrichment of *Clostridiales* and *Enterococcus* members seen at 72 hours (compared with admission).[8] In another clinical study, the β diversity on admission in severely injured patients was predictive of clinical outcomes.[9] A previous study by Nicholson et al.,[10] observed that the gut microbiome was altered in as little as 30 minutes from the time of injury and that a subpopulation

of injured trauma patients who had received larger amounts total blood products demonstrated smaller shifts in diversity when compared with controls, suggesting that early massive transfusion is associated preservation of species diversity.[10]

While the aforementioned results display an association with resuscitation volumes and gut microbiome changes, causality was not addressable. The objectives of this study were to establish the role of gut hypoperfusion and changes in the gut microbiome following traumatic injury and to assess if there is a benefit of early whole blood (WB) resuscitation in maintaining the gut microbiome using a preclinical multiple injuries hemorrhage model.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee of the US Army Institute of Surgical Research. Research was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council. The facility's Institutional Animal Care and Use Committee approved all research conducted in this study. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Sprague-Dawley rats (350–450 g) were anesthetized with isoflurane and 100% oxygen. The left femoral artery and vein were cannulated for measurement of arterial blood pressure and to allow for blood sampling and manually controlled hemorrhage. Rats were anesthetized throughout the entirety of the experiment and euthanized at 2 hours.

A well-established, highly standardized rat model of severe multiple injuries and hemorrhage developed at the US Army Institute of Surgical Research was used.[11] Briefly, all rats underwent a midline abdominal incision through the skin and underlying muscles to gain access into the abdomen. Multiple injuries were induced by crush injuries to the left and medial liver lobes and the right leg skeletal muscle and by fracture of the right femur. In the hemorrhage group, rats were then immediately bled to 40 mm Hg and held there until 40% of the estimated blood volume was removed. Once hemorrhage was completed, the blood pressure and heart rate were allowed to freely compensate for 1 hour. In the resuscitation group, fresh WB (20% of estimated blood volume) was collected in citrate phosphate dextrose solution (at 1:8 ratio) from the femoral artery of an anesthetized donor rat and transfused at 1 hour posttrauma (Fig. 1).

Rats were randomly assigned to one of four groups, allowing the groups to be distributed evenly across all litters of animals. Sham rats (S, n = 6) underwent laparotomy only, which allowed for measurement of changes in cecal oxygen concentration in the absence of multiple injuries (i.e., due to anesthesia). Rats assigned to the second experimental group underwent the previously described multiple injuries (T, n = 5). The third experimental group consisted of multiple injuries combined with 40% hemorrhage (H, n = 7). Rats assigned to the resuscitation group underwent multiple injuries and 40% hemorrhage followed by transfusion of fresh WB at 1 hour postinjury (R, n = 7) (Fig. 1).

Sample Collection

Blood chemistry (iSTAT, Abbott, Princeton, NJ) and blood counts (ADVIA, Siemens Healthcare Diagnostics Inc., Japan) were measured from venous blood samples collected at baseline (2 mL) and at 1 hour (1 mL) and 2 hours (2 mL) postinjury. Baseline and 1 hour samples (3 mL total) were counted toward the total volume of hemorrhage. Fecal samples were collected at baseline (from the induction chamber) and then at necropsy from the rectum. Of note, the microbiome changes along the length of the intestine and collecting from the rectum allowed for consistency between time points. The fecal samples were frozen at −80°C and the QIAmp PowerFecal Pro DNA Kit (Qiagen, Germantown, MD) was used to purify DNA from all fecal samples (~250 mg of fecal material used). DNA was quantified using a NanoDrop (ThermoFisher Scientific, Waltham, MA) spectrophotometer before amplification. Fecal samples were purified and sequenced in two batches; the first four rats from each group were sequenced in *batch 1*, with the remaining animals completed in *batch 2*. Although the same isolation kits and reagents were used, the library preparation between batches differed. Batch effect was accounted for in all statistical analyses.

Using a PreSens Microx 4 (PreSens Precision Sensing, Regensburg, Germany), a portable fiber optic microsensor, we directly measured the oxygen concentration of the cecal mesentery at baseline and at 1 hour and 2 hours postinjury. The measurement at the 1 hour time point was before the administration of WB in the resuscitation group.

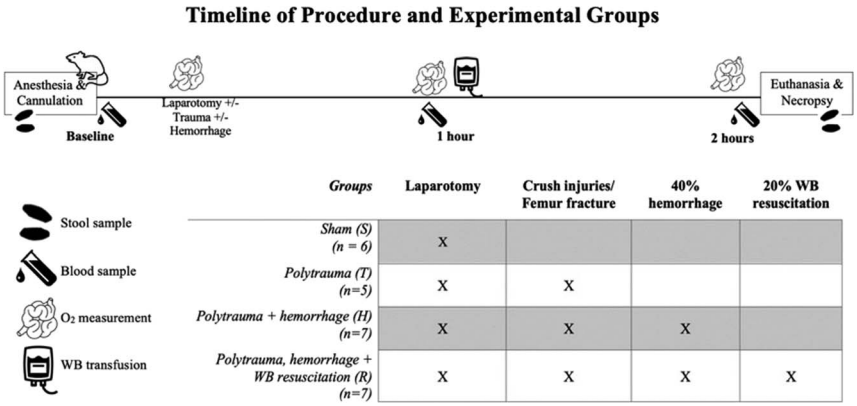

Figure 1. Timeline of procedure highlighting time points of sample collections, measurement of mesenteric oxygen concentration, and WB resuscitation and experimental groups.

Microbiome Analysis

The V3-V4 variable region of the 16S rRNA gene was amplified from genomic DNA with primers U341F (CCTACGGGRSGCAGCAG) and Bakt_805R (GACTACHVGGG TATCTAATCC).[12] Sequencing libraries were prepared using the Nextera XT Index Kit v2 and sequenced on the Illumina MiSeq platform with the 600-cycle MiSeq Reagent Kit v3 (Illumina, San Diego, CA). An average of 79,997 nonchimeric, pair-end reads were generated for each sample. 16S rRNA gene sequences were processed as described previously using the QIIME2 (version 2019.1, Knight Lab, La Jolla, CA; Caporaso Lab, Flagstaff, AZ) pipeline.[13,14]

Statistical Analyses

All data were analyzed in either Graph-Pad Prism (version 8.1.2, GraphPad Software, San Diego, CA) or R (version 3.5.1, R: The R Foundation, Free Software Foundation, Inc., Boston, MA). To account for batch effects, each response variable was fitted to separate linear mixed-effects models with varying intercepts among batch and subject within batch. For α diversity, each metric (operational taxonomic units richness, and Shannon, Pielou, and Faith diversities) served as response variable. For β diversity, betadisper was used to evaluate within-group dispersions. Batch effect was included in a nonparametric multivariate model in Adonis.[15] For differential abundance analysis, raw taxonomic counts were first filtered for low abundance taxa (0.01%) and then centered log ratio transformed using the mixOmics package in R.[16] Centered log ratio transformed data were analyzed using separate linear mixed-effects models incorporating the Benjamini-Hochberg procedure to adjust for false discovery. For biomarker identification using linear discriminant analysis effect size, the centered log ratio transformed data were corrected for batch effects using the ComBat function in the sva package.[17,18] Significant effects were evaluated at $\alpha = 0.05$.

The analysis of physiologic data and biochemistry parameters was performed by SigmaPlot (Systat Software, Inc., San Jose, CA). The two-way repeated analysis of variance was used to test the mean difference among the groups at each given time point or for the continuous variables over study time points within each group followed by a pairwise comparison using a Tukey method. Data are presented as means ± standard error of the mean, and statistical significance was accepted at the $p < 0.05$.

RESULTS

Hemodynamic Changes

Multiple injuries and hemorrhagic shock led to a significant decline in mean arterial pressure at 1 hour and 2 hours after

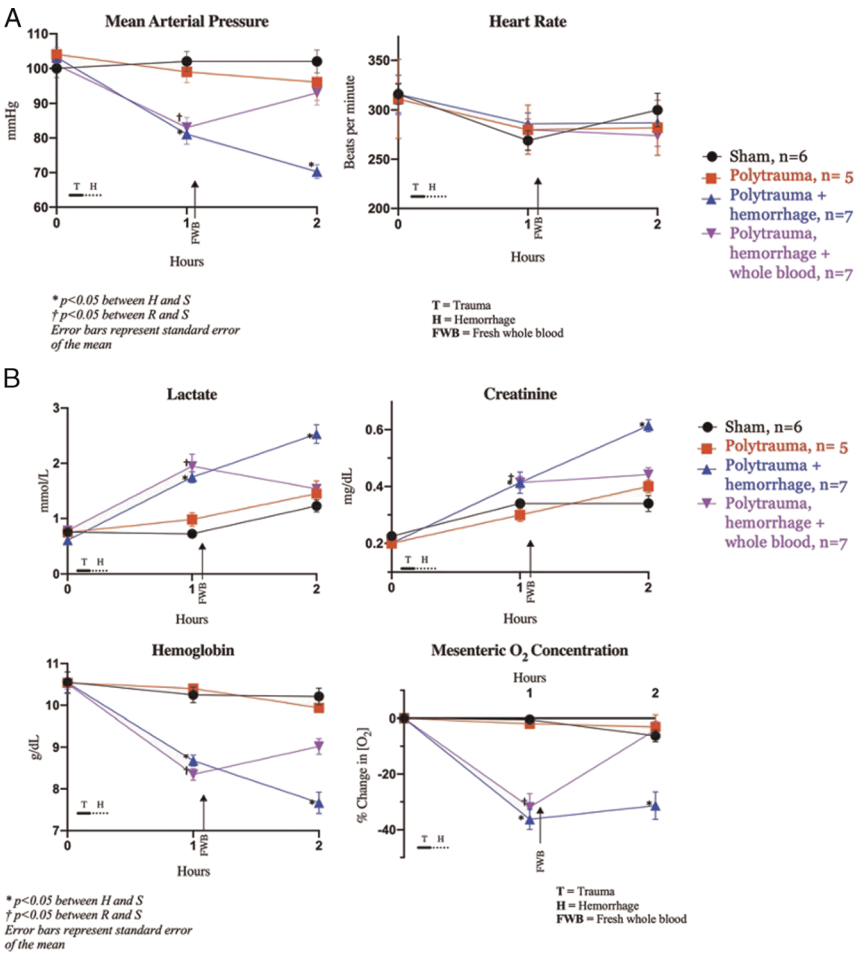

Figure 2. (*A*) Hemodynamic changes, illustrated by mean arterial pressure and heart rate. (*B*) Perfusion and end organ measurements, illustrated by changes in lactate, creatinine, and hemoglobin and changes in mesenteric oxygen concentration.

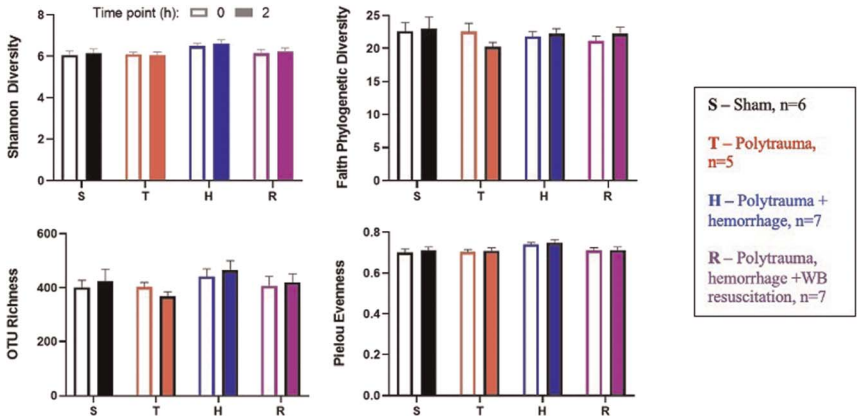

Figure 3. α Diversity preinjured (open bars, 0 hours) and postinjured (filled bars, 2 hours) rats as measured by Shannon diversity, Faith phylogenetic diversity, operational taxonomic units richness, and Pielou evenness. No significant difference across groups or time was observed. Bars depict the mean and standard error of the mean.

injury. Limited resuscitation with 20% fresh WB improved mean arterial pressure at 2 hours to within 10% of baseline. At 2 hours, there was no significant difference in mean arterial pressure among the S, T only, or R rats, which were all significantly higher than the rats in the H group (Fig. 2A).

Physiologic Parameters

Lactate was only slightly increased in the S and T groups at 2 hours compared with baseline, with no significant difference found between the two groups. A two-way analysis of variance revealed a significant increase in lactate at 1 hour following hemorrhage. Lactate continued to rise in the H rats, higher (2.5 ± 0.17) at 2 hours compared with S (1.2 ± .10), T (1.5 ± 0.23), or R (1.5 ± 0.14) rats ($p < 0.05$) (Fig. 2B).

There was a similar significant increase in creatinine at 1 hour after hemorrhage, which also continued to increase in the H rats, significantly higher than all other groups at 2 hours (Fig. 2B).

A significant decline in hemoglobin was observed at 1 hour in all rats that underwent multiple injuries and hemorrhage (H and R rats) with a continued decline at 2 hours in the H rats, which were significantly lower than all other groups at 2 hours. Alternatively, in R rats, there was an increase in hemoglobin at 2 hours; however, it did not return to baseline (Fig. 2B).

Tissue Oxygen Concentration

Multiple injuries combined with hemorrhage resulted in a significant decrease in the oxygen concentration of the cecal mesentery at 1 hour postinjury compared with baseline. In the multiple injuries hemorrhage group, there was an average decline of 36% ± 3.6% at 1 hour, without significant recovery at 2 hours. In the group that received fresh WB, a similar decline (32% ± 4.9%) at 1 hour was observed; however, the tissue oxygen concentration was restored to near baseline by 2 hours postinjury. There was no significant decline in the tissue oxygen concentration in the sham or multiple injuries only rats at 1 hour or 2 hours compared with baseline. At 2 hours, there was no significant difference in the percent change of oxygen

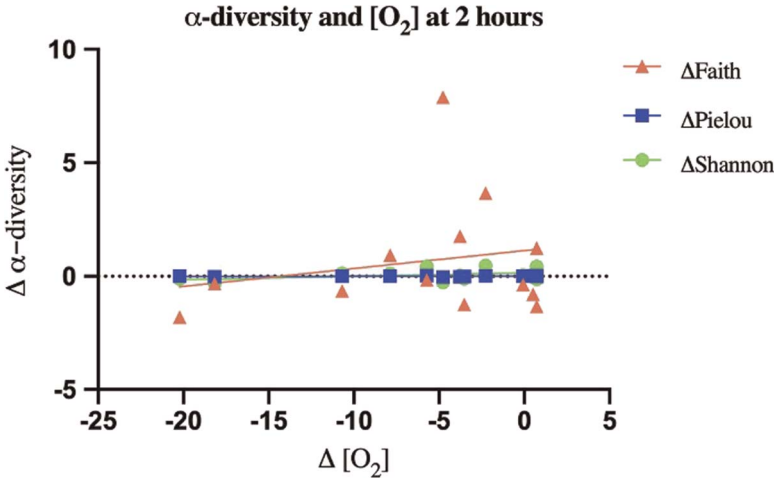

Figure 4. Scatter plot showing the association between changes in α diversity and changes in mesenteric oxygen concentration at 2 hours for hemorrhaged and resuscitated rats.

concentration among the sham, multiple injuries only, and resuscitated group, but a significant difference in percent change was seen between these groups and the hemorrhage group (Fig. 2B).

Bacteria α and β Diversity

The α diversity, or within-sample diversity, as measured by Shannon and Faith phylogenetic diversity indices, operational taxonomic units richness, and Pielou evenness, was not significantly different between groups or within treatment groups over time, demonstrating that microbial species diversity was unchanged by multiple injuries, hemorrhage, and resuscitation at 2 hours postinjury (Fig. 3). However, we did find a correlation between change in α diversity and change in oxygen concentration when comparing the H and R rats. There was a positive relationship with change in mesenteric oxygen concentration and α diversity at 2 hours (Fig. 4).

Conversely, β diversity, or between-sample diversity, differed among all groups for all measured indices but did not reach significance in the Bray-Curtis index. The principal coordinates analysis plots for these variables and their significance levels are shown in Figure 5. Importantly, there were no differences in the dispersion among groups, suggesting that the significant differences among groups for β diversity was due to the spatial median (location differences). To further explore the difference in spatial medians between groups, we performed a pairwise contrasts. At 2 hours, there were significant differences in β diversity between the S and H rats across all measured indices, with no significant difference seen between the S and R rats, indicating that WB resuscitation may serve to mitigate alterations in microbial diversity following trauma and hemorrhage.

Sequence reads were classified at the phylum to genus level. The dominant phyla in both the preinjured and postinjured groups were *Firmicutes* followed by *Bacteroidetes*, but no

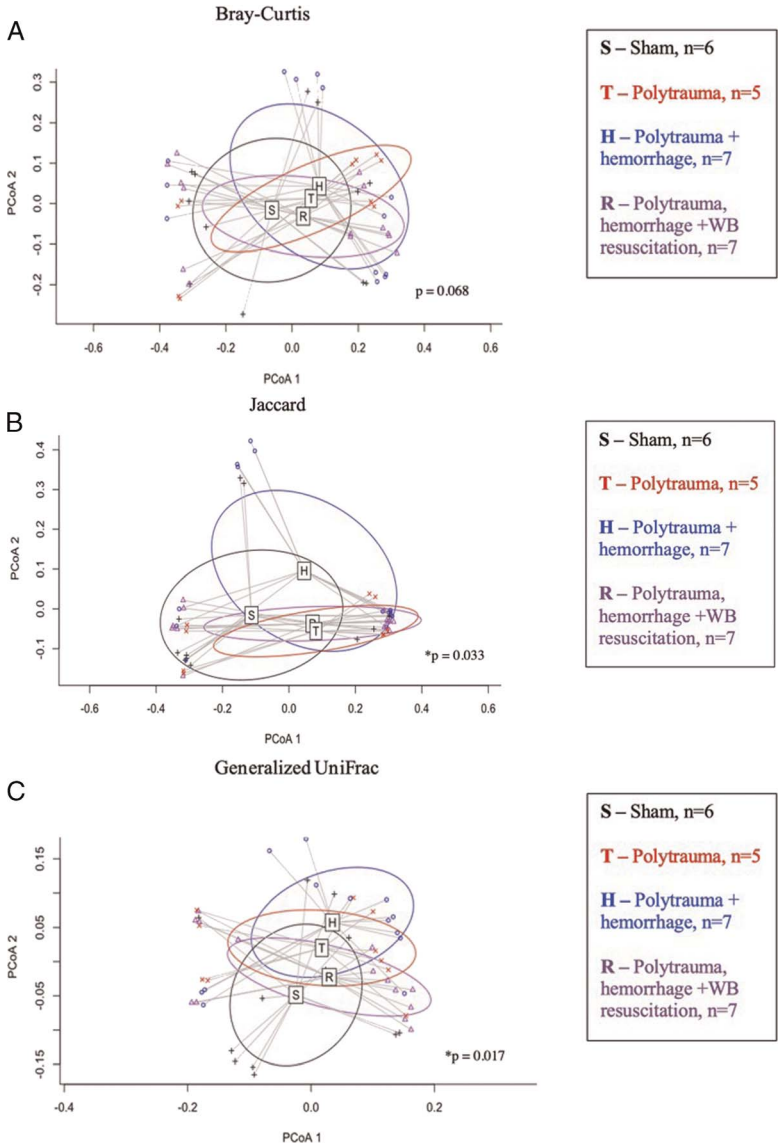

Figure 5. Principal coordinates analysis plots for β diversity as measured by (*A*) Bray-Curtis, (*B*) Jaccard, and (*C*) generalized UniFrac. β Diversity differed for all measured indices but did not reach significance in Bray-Curtis; group effect *p* values are noted within each plot.

significant differences at the phylum level were seen over time or by group. However, at the genus level, we identified 14 genera that were differentially abundant between baseline and postinjury across all groups (Fig. 6). Notable among these were increases in *Roseburia* and *Enterobacteriaceae* along with decreases in *Rothia* and *Streptococcus.*

DISCUSSION

The gut microbiome has long been postulated as a source of systemic metabolic derangement. Advancements in DNA sequencing and bioinformatics have allowed for an enhanced understanding of the microbial communities within the human intestinal tract and its function in hemostasis.[4,19] Over the past decade, attention has been given to its role and disruption in critical illness and injury. Until recently, less defined was the impact of traumatic injury on the composition of a patient's gut microbiome and the consequences of dysbiosis in this population. Recent human studies by our group have demonstrated several key findings in the gut microbiome following severe injury —the gut microbiome is altered in within 30 minutes following injury in adults, with dysbiosis occurring throughout the hospital stay; recipients of large amounts of blood products had a gut microbiome that more closely resembled that of healthy controls and demonstrated increased species diversity with more units transfused; and the gut microbiome on admission is associated with clinical outcomes including hospital and ICU length of stay, ventilator days, and acute respiratory distress syndrome.[9,10]

To establish the effect of transfusion volumes and microbiome changes, we used a preclinical model to detect changes in the gut microbiota following multiple injuries, hemorrhagic shock, and WB resuscitation. One novel aspect of our study design was the ability to measure intestinal perfusion via direct measurements of tissue oxygen concentration. A PreSens Microx 4, a portable fiber optic microsensor, was used to measure the oxygen concentration of the cecal mesentery at baseline and at 1 hour and 2 hours postinjury. Limited WB resuscitation was found to restore the tissue oxygen concentration of the cecal mesentery following 40% hemorrhage. At 2 hours, mesenteric oxygenation in the resuscitated animals (that had previously undergone 40% hemorrhage) was similar to animals that did not undergo hemorrhage. These changes can be thought of in relation to organ perfusion as well, since similar trends following WB resuscitation were observed for mean arterial pressure, lactate, creatinine, and hemoglobin. Limited resuscitation restored mean arterial pressure to within 10% of baseline by 2 hours. In addition, at 2 hours, lactate and hemoglobin in the resuscitated group were at levels similar to the nonhemorrhaged animals. These trends were also observed in prior studies by our group.[11,20]

We did not observe significant changes in α diversity between groups or within treatment groups, consistent with our prior preclinical rat studies.[21] However, at 2 hours, we did see a positive relationship between change in α diversity and mesenteric oxygen concentration when comparing the hemorrhaged and WB resuscitated rats. A positive correlation indicates that microbial species diversity is enhanced with improvements in mesenteric perfusion. Although it reached significance, the R^2 was low, indicating a poor fit to this model, which would be further improved by increasing experimental power. However, changes in α diversity are not as frequent in this acute time frame, and the correlation with blood flow indicates that we may eventually see differences in this metric with longer follow-up time points. Significance in the β diversity among all groups was observed, indicating that gut microbial diversity is influenced by trauma and interventions affecting perfusion. Insults resulting in hypoperfusion and intestinal ischemia reperfusion not only increase intestinal permeability, allowing for translocation of bacteria and their toxins into the systemic circulation, but also destroy commensal bacteria,[6,7] leading to greater derangements in the gut microbiome. Previous clinical studies also found that β diversity differed according to the amount of blood products transfused to patients following trauma.[10] Through pairwise contrast, we saw a significant difference in the spatial medians of the hemorrhaged animals versus sham

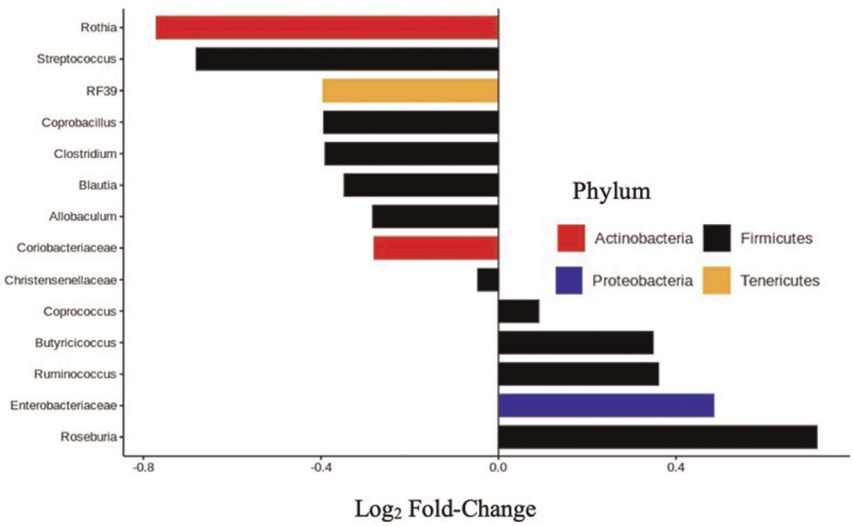

Figure 6. Bar plot showing the log2 fold change of operational taxonomic units that differed significantly at 2 hours relative to baseline. Each bar depicts the genus or the highest order of taxon that could be classified (i.e., family or order) with bar colors indicating the phylum to which each taxon belongs.

animals. In contrast, the animals that were resuscitated had spatial medians more similar to the sham group. While further studies are needed to better delineate changes in the gut microbiome following trauma, hemorrhage, and resuscitation, these early results suggest that resuscitation with WB may mitigate the impact of hemorrhage and hypoperfusion on gut microbial diversity.

Although we did not see significant changes in phyla preinjury and postinjury, we did observe changes at the genus level over time. At 2 hours, increases were seen in *Roseburia* and *Ruminococcus*, both members of the phylum Firmicutes, traditionally viewed as "health-promoting" bacteria. This was coupled with decreases in *Rothia* and *Streptococcus*, both from traditionally beneficial phyla.[22] Conversely, at 2 hours, there was also an increase in *Enterobacteriaceae*, traditionally recognized as pathogenic bacteria that include such organisms as *Escherichia coli*, and members of *Klebsiella*, *Salmonella*, and *Shigella*. While increased experimental power is needed to further define taxonomic differences between treatment groups, these data reillustrate that multiple injuries alter microbial abundance, leading to dysbiosis. Rapid dysbiosis is seen following traumatic injury and in critical illness and worsens during prolonged hospitalization.[5,10] Dysbiosis has also been attributed to septic complications in critically ill patients, likely because of the key role that symbiont organisms play in colonization resistance against acquired pathogens.[22,23]

Gut microbial communities shape human health and disease and regulate immune status.[24–26] Functional changes to gut microbial communities, such as short chain fatty acid production, can further affect the host immune system. A healthy microbiome is considered to have a high abundance of butyrate-producing bacteria. Loss of butyrate producing microbes can disrupt the host response to infection or injury.[27,28] In addition, the gut microbiota can prevent pathogen colonization through "competitive exclusion," in conjunction with the production of antimicrobial substances and the stimulation of mucus and immunoglobulin A production, which serve to strengthen the intestinal barrier.[29,30] Intestinal ischemia reperfusion has been implicated in the pathophysiology that contributes to gut microbial alterations. Damage to the epithelial barrier of the colon directly follows intestinal ischemia reperfusion and leads to dysbiosis.[6] Intestinal ischemia resulting from hemorrhagic shock may lead to alterations in colonocyte energy metabolism and depletion of health-promoting anaerobes, thereby facilitating pathogen colonization and infection. Assessment of the functional changes to the gut microbiome represents future directions to better study the implications of trauma- and hemorrhage-associated dysbiosis on outcomes. The disruption of the anatomic and functional integrity of the gut and the associated health community of gut microbes can result in systemic inflammation, bacterial translocation, and sepsis, all of which may influence clinical outcomes following traumatic injury.

Traumatic injury and the associated hemorrhagic shock and gut hypoperfusion may ignite a cascade of events starting with gut dysbiosis that then predisposes injured patients to inflammatory and infectious complications resulting in worse outcomes. Given the essential role of the gut microbiome in the immune response and maintenance of health, the implications of dysbiosis following traumatic injury and hospitalization on clinical outcomes are profound. If the gut microbiome is unable to maintain resiliency in the context of traumatic injury and critical illness, patients are at an increased risk for development of a pathobiome and subsequent inflammatory- and infection-related complications. Early prehospital monitoring of markers that can provide identification of the need for WB transfusion holds the potential to mitigate dysbiosis and diminish the effects of a pathobiome by intervening at an early and crucial time point. Furthermore, since the gut microbiome is a potentially modifiable factor through therapeutic intervention or replacements, this study lays the groundwork for future investigations to explore these therapeutic options. Currently, fecal microbiota transplant has been used in treating *Clostridium difficile* colitis and inflammatory bowel disease, and therapies such as fecal microbiota transplant could be modified for trauma patients.[31,32] Other potential therapies include prebiotic-, probiotic-, and synbiotic-targeted nutritional strategies.

There were several limitations to our study, namely, the sample size per group, as the latter part of our study was halted because of the COVID-19 pandemic. It is likely that by increasing our sample size in future studies, the time × group interaction will become significant, allowing us to further delineate change in the microbiota over time by treatment group. A prior study by our group showed changes in the rat microbiome in as little as 2 hours,[21] and our study endpoint was set at 2 hours. However, a longitudinal analysis may also reveal additional alterations in the gut microbiome beyond this time point. As mentioned, DNA purification and sequencing were completed in two batches (not uncommon to microbiome studies), which led to a significant batch effect that was accounted for in modeling and statistical analysis. However, methods to adjust are overly aggressive and adversely impact the power of the study; this will be addressed in further studies. Other techniques to consider for future studies include the use of whole genome sequencing (vs. targeted 16S sequencing), which would allow for a more inclusive capture of changes in the microbiome, and shotgun metagenomic sequencing to also identify functional differences associated with changes in the microbiome.

In conclusion, gut hypoperfusion following traumatic injury influences diversity of the gut microbiome. Whole blood resuscitation not only restores mesenteric perfusion but also may mitigate the detrimental effects of hemorrhage on intestinal dysbiosis, thereby providing benefit beyond the initial hemodynamic and hemostatic properties. The level of dysbiosis soon after trauma may serve as a (1) novel metric of hypoperfusion-mediated gut injury, (2) an independent predictor of outcomes, (3) a potential therapeutic target to influence fluid requirements, or (4) an endpoint to adequate resuscitation. Since the gut microbiome is modifiable, microbiome replacements hold potential value for treating injured patients.

AUTHORSHIP

J.Y. contributed in the design, data acquisition and analysis, and interpretation of data. W.M. contributed in the analysis and interpretation of data. X.W. contributed in the design, data acquisition and analysis, and interpretation of data. D.B. contributed in the design and analysis, and interpretation of data. D.D. contributed in the design and analysis, and interpretation of data.
D.Z. contributed in the data acquisition. Z.L. contributed in the data acquisition. S.S. contributed in the data acquisition. A.P.C. contributed in the design and analysis and interpretation of data.

J.B. contributed in the design and analysis and interpretation of data. S.E.N. contributed in the design, data acquisition, and analysis and interpretation of data.

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DISCLOSURE

The authors declare no conflicts of interest.

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